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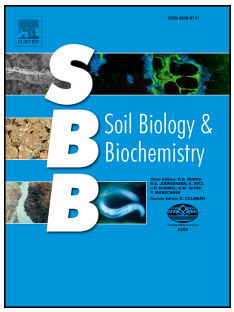

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Nitrogen addition alters composition, diversity, and functioning of microbial communities in mangrove soils: an incubation experiment

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Abstract

Mangrove ecosystems are important for carbon storage due to their high productivity and low decomposition rates. Waterways have experienced increased nutrient loads as a result of anthropogenic activities and it is unclear how this may affect carbon and nutrient cycles in downstream mangroves that receive these nutrient-rich waters. Using a laboratory-based incubation experiment, this study aimed to assess the effects of nutrient addition on the diversity and structure of mangrove soil bacterial communities, as well as biomass and activity of the soil microbial community, under different oxygen conditions. Bacterial community diversity and composition was characterised using 16S rRNA gene sequencing and microbial activity was examined through the measurement of microbial respiration and the activities of enzymes associated with organic matter decomposition. Nitrogen addition caused clear shifts in bacterial community composition, with decreases in bacterial diversity and the abundance of sulfate-reducing bacteria. Microbial biomass also decreased with nitrogen addition under reduced oxygen incubations. Changes in bacterial community structure were accompanied by changes in the activity of some enzymes involved in carbon, nitrogen and phosphorus cycling. Under reduced oxygen conditions, nitrogen addition resulted in a significant increase in the microbial metabolic quotient but no accompanying change in microbial respiration, which was explained by a decrease in microbial biomass. The findings of this study indicate that nitrogen loading has potential implications for microbial communities and carbon and nutrient cycling in mangrove environments that warrant further investigation under field conditions.

Keywords: mangroves, coastal wetlands, nutrient pollution, bacterial diversity

1. Introduction

Nitrogen (N), phosphorus (P), and potassium (K) are essential nutrients for plants and microbes, and limitations in these nutrients can constrain growth and functioning. Anthropogenic activities have altered fluxes of these growth-limiting nutrients in freshwater and coastal marine ecosystems (Smith 2003), which can have detrimental impacts on water quality and the organisms that live in these environments (Smith et al. 1999). In many tropical and sub-tropical coastal regions, mangrove forests are often found in

estuaries at the receiving end of nutrient enriched riverine waters. As mangrove ecosystems are important coastal carbon (C) stores (Donato et al. 2011, Alongi 2012), there is a need to better understand how elevated nutrient levels may impact ecosystem functioning, such as C and nutrient cycling, in these environments.

Several field experiments have assessed mangrove plant responses to N and P addition. Generally, they have found that when plants were given a limiting nutrient, growth increased significantly, both aboveground (Feller 1995, Feller et al. 2003, Lovelock et al. 2004, Dangremond et al. 2019) and belowground (McKee et al. 2007), which in turn should increase leaf and root litter inputs to the soil. Chemical and structural changes have also been observed in the leaves of these plants, with reduced sclerophylly (leaf toughness), and altered concentrations of nutrients (Keuskamp, et al. 2015a, Simpson et al. 2020) and phenolic compounds with fertilisation (Feller 1995; Feller et al. 1999; Keuskamp et al. 2015a; Keuskamp et al. 2015b). Increases in litter quantity and changes in litter quality could have implications for soil processes, particularly those related to the C cycle (e.g. rates of decomposition, C storage and surface accretion). Further, nutrient enrichment will likely impact mangrove soil bacterial communities, which play an important role in C flux processes in mangrove soils (Holguin et al. 2001), and account for most of the total soil microbial biomass (Chen et al. 2016) in these systems. Despite this, few studies have investigated the impacts of nutrient enrichment on mangrove soil bacterial communities.

Several recent meta-analyses and large-scale multi-site studies have assessed overall trends of soil microbial responses to nutrient enrichment across a range of terrestrial ecosystems. Nitrogen addition has been associated with decreases in soil microbial diversity (Wang et al. 2018) and microbial biomass (Treseder 2008, Ramirez et al. 2012), while P addition can increase soil microbial biomass (Yue et al. 2018). Many of these terrestrial studies have also observed changes in soil bacterial community composition with nutrient enrichment (Leff et al. 2015, Ramirez et al. 2012, Wang et al. 2018), which may contribute to observed changes in soil functioning. Soil respiration responses to N and P addition vary across ecosystem types and have been associated with changes in microbial biomass, root biomass and soil C (Zhou et al. 2014, Feng & Zhu 2019). Phosphorus addition has also been shown to suppress activity

of P acquiring enzymes (Marklein & Houlton 2012, Xiao et al. 2018) and N addition can stimulate C and P acquiring enzymes, but suppress lignin-modifying oxidase activity (Chen, Luo et al. 2018, Xiao et al. 2018). This decrease in oxidase activity, as well as increased litter input, can result in increased soil C storage in N-amended soils (Chen, Luo et al. 2018).

Despite differences in environmental conditions compared to terrestrial soils, similar microbial responses to nutrient addition have been observed in mangrove and marine environments. Nitrogen addition to mangrove and marine sediments was found to decrease microbial biomass (Keuskamp et al. 2015b), bacterial abundance (Luo et al. 2017), and bacterial diversity (Aoyagi et al. 2015; Wang at al. 2016). The redox conditions and low N levels in mangrove soils mean that the majority of the organic matter decomposition in mangroves occurs via sulfate reduction (Kristensen et al. 1991). However, studies that have experimentally increased levels of the more thermodynamically favourable nitrate have observed increases in the abundance of Sulfurimonas (Aoyagi et al. 2015, Wang et al. 2016), nitrate-reducing genus of bacteria. These shifts in soil bacterial communities could lead to changes in soil functioning, as well as increased emissions of greenhouses gases including carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4), as has been observed in some studies (Muñoz-Hincapié et al. 2002, Kreuzwieser et al. 2003, Chen et al. 2011). Changes in potential enzyme activity have also been observed in response to nutrient addition (Tam 1998, Luo et al. 2017), although responses can vary between vegetation zones within the mangrove ecosystem (Keuskamp et al. 2015b). When combined with changes in plant productivity, these nutrient-induced changes in soil functioning could affect C cycling and decomposition processes leading to changes in both the rate and direction of surface elevation change (McKee et al. 2007), an important factor when considering resistance and resilience of these ecosystems to rises in sea level.

The aim of this experiment was to assess the effects of N addition alone, and in combination with P and K, on the diversity, structure and function of mangrove soil microbial communities, with a focus on bacterial communities, being the dominant component of the microbial biomass of mangrove soils (Chen et al. 2016). A laboratory-based, controlled soil incubation experiment enabled us to focus on soil microbial

98 responses to nutrient enrichment by removing plant-mediated responses and potential impacts of other
99 fluctuating environmental variables on microbial communities. We sought to identify which bacterial
100 groups change with nutrient addition, and to link this with observed changes in microbial functioning. As
101 mangrove soils experience both aerobic and anaerobic conditions, each with their own distinct microbial
102 communities, we incubated soil in two different oxygen environments to compare responses. This
103 information will improve our understanding of microbial responses to elevated nutrients and the
104 potential implications for C storage and nutrient cycling in mangrove environments.

105 This study was conducted with soils collected near a long-term field fertilisation experiment in northeast
106 Florida where plant growth responses indicate that the site is limited by N, but not P (Dangremond et al.
107 2019). However, concentrations of leaf N:P in the control plots (Simpson et al. 2020) suggest that plant
108 growth could be limited by either N, P, or both (Koerselman & Meuleman 1996). A decomposition study
109 at the site found initial decay rates of leaf litter were higher in the nutrient addition treatments but
110 stabilised across all treatments within six months (Simpson et al. 2020). The soil microbial communities of
111 this site had not yet been investigated, but we hypothesised that N addition would: (1) decrease bacterial
112 diversity, and (2) decrease abundance of sulfate-reducing bacteria. In terms of soil function, we
113 hypothesised that N addition would: (3) increase microbial respiration and the activity of a P acquiring
114 enzyme, and (4) decrease activity of lignin-modifying enzymes and an N acquiring enzyme. If the
115 microbial community is also P and/or K limited, the addition of PK in combination with N could have
116 additive effects on microbial responses. However, if the microbes are also limited by available C sources,
117 the response to nutrient addition may be limited.

118 **2. Methods**

119 *2.1 Study site and soil sampling*

120 The soil for this experiment was obtained from Guana Tolomato Matanzas National Estuarine Research
121 Reserve, St John's County, Florida (29.7291°N, 81.2415°W). The site is located within the saltmarsh-
122 mangrove ecotone near the northernmost range limit of mangroves in Florida (Giri & Long 2016) and
123 consists of black mangrove (*Avicennia germinans*) shrubs amongst *Batis maritima* and *Sarcocornia*

perennis saltmarsh plants. Soil was collected in April 2018 at low tide when the soil surface was exposed. Approximately 20 L of soil was collected into a clean bucket using a trowel to a depth of 10 cm underneath unfertilised *A. germinans* shrubs, across an area of approximately 100 m^2. Oxygen concentrations rapidly decline with depth from the surface in mangroves (Booth et al. 2019) and we targeted the top 10 cm to ensure we collected a mix of aerobic and anaerobic communities. Due to the peaty nature of the soil, instead of sieving it was sorted by hand in the lab to remove leaf litter, woody roots, sticks, shells, and other large debris, and then thoroughly mixed to homogenise. Soil was homogenised to minimise variation amongst mesocosms prior to nutrient application so differences at the end of the experiment could be attributed to treatments.

2.2 Incubation experiment

Soil mesocosms were set up in 473 mL (16 oz.) Ball® smooth sided jars (Rubbermaid Inc., Atlanta, GA, USA) with approximately 72 g (fresh weight) of the homogenised soil pressed down into the bottom of the jar. The lids of the jars were modified with stop cock valves and gas tubing to enable gas measurements. Soils were processed and weighed into jars on the day of collection. Mesocosms were randomly assigned to one of four nutrient treatments (control, +N, +PK, +NPK), and one of two oxygen treatments (ambient or reduced oxygen incubation) in a fully factorial design, with five replicates for each treatment combination (total n = 40). To obtain a reduced oxygen environment, jars were flushed with N_2 gas and the valves in the lid were then closed. Ambient oxygen jars were lidded, but with the valves left open. The mesocosms were left to incubate for four days to allow the soil to settle prior to administering the nutrient treatments.

Soil samples collected from the site in December 2017 indicated background soil concentrations of approximately 10.8 g/kg total N and 0.387 g/kg total P on a dry weight basis. This experiment aimed to double those concentrations by adding 2 mL of 4.95 M ammonium nitrate (NH_4NO_3) solution as the N source, and 1 mL of 0.16 M potassium phosphate dibasic (KH_2PO_4) as the source of P and K. Deionised water was added to mesocosms as required to ensure that 3 mL of liquid was held constant in each treatment. The liquid was mixed into the soil to ensure even distribution, then the lids were refitted and

the reduced oxygen mesocosms flushed again with N_2 gas. The average soil moisture content (wet weight basis) of three samples taken from the homogenised soil before the start of the experiment was 82.2% and addition of the nutrients in liquid form would have increased this to ~82.9%. The mesocosms were kept in a dark room at around 22 °C when not being measured which is within the temperature range that would be experienced in the field at that time of year. The mesocosms were kept in the dark to prevent growth of algae or seeds ensuring that responses could be linked predominantly to microbial communities. To maintain soil moisture, after 11 days deionised water was added to each mesocosm until the starting weight was reached. The experiment ran for 15 days in April 2018, and all mesocosms were then destructively harvested for soil analyses.

2.3 Soil analyses

Soil pH was measured with an accumet pH meter (Fisher Scientific, Pittsburgh, PA, USA) in a 1:2 fresh soil to deionised water slurry. Total soil C and N were measured by dry combustion of dried, ground soil using a Vario EL cube CN analyser (Elementar Analysensysteme GmbH, Hanau, Germany). Soil moisture content was calculated gravimetrically on a wet weight basis from soil that had been oven-dried at 105 °C for 48 hours. Organic matter content and total soil P were determined using a loss on ignition (LOI) method (Kuo 1996) by combusting soils at 550 °C for 4 hours then using 0.5 M H_2SO_4 as an extractant. Phosphate in the acid extracts was measured colourimetrically on a CLARIOstar microplate reader (BMG LABTECH, Ortenberg, Germany) using an ascorbic acid method adapted from Kuo (1996), with volumes scaled down for use in a microplate. All results are presented as means ± 1 standard error.

2.4 Microbial biomass and activity

Microbial biomass C was determined for each sample using the chloroform fumigation-extraction method (Vance et al. 1987) with 0.5 M K_2SO_4 as an extractant. The standard 24 hour fumigation time was used as this has previously been identified as suitable for water-saturated soils from rice fields (Witt et al. 2000). Total C was measured in the extracts with a TOC-L analyser (Shimadzu, Kyoto, Japan) and an extraction efficiency coefficient of 0.45 (Joergensen et al. 2011) was applied to calculate microbial biomass C.

CO_2 flux was measured daily with an LI-8100A automated soil CO_2 flux system (Li-Cor Inc., Lincoln, Nebraska, USA) to assess microbial respiration. The first measurement from each mesocosm was taken within 30 minutes of the addition of the nutrients (or water) and each morning thereafter. Four consecutive flux readings of 60 seconds were measured. The initial reading was not included in the analysis to allow sufficient time for flushing of the gas lines and an average was calculated from the last three readings. All reduced oxygen mesocosms were flushed with N_2 gas immediately prior to measurement to flush out any respired gases that had accumulated while sitting. The ambient oxygen mesocosms had their lids removed for a few minutes prior to analysis and then refitted immediately prior to measurement to ensure that ample fresh air was getting into the mesocosms. When water was added mid-experiment to the mesocosms to maintain soil moisture content, the reduced oxygen mesocosms required more water than the ambient mesocosms, which was likely due to the daily flushing of N_2 gas. This led to a significant change in CO_2 flux readings in the reduced oxygen mesocosms that was not observed in the ambient mesocosms. For this reason, we excluded the flux readings made after that point from the respiration analyses for both oxygen treatments. One of the ambient oxygen +PK samples was removed from the respiration analysis as it had unusual readings for the first few days, most likely due to a suspected air leak in the lid during measurements. The metabolic quotient (qCO_2) was calculated as the ratio of microbial respiration to microbial biomass C on the final day of the experiment (Anderson & Domsch 1985).

Potential extracellular enzyme activity was measured in soil that had been stored at 4 °C using assays for two oxidative enzymes (lignin peroxidase (PER) and phenol oxidase (POX)), and six hydrolytic enzymes (urease (URE), cellobiohydrolase (CBH), *β*-*N*-acetylglucosaminidase (NAG), acid phosphatase (AP), xylosidase (XYL) and *β*-glucosidase (GLC)). All enzyme activity assays were performed within two weeks of sampling following the methods from Jackson, Tyler, & Millar (2013) for *p*NP-linked substrates (CBH, NAG, AP, XYL and GLC) and Cordero, Snell & Bardgett (2019) for URE. L-DOPA assays were used to evaluate POX and PER activity following the methods in De Long et al. (2019) with double the amount of fresh soil added to the soil slurry due to high soil moisture content. Saturating substrate concentrations for *p*NP-linked substrates and URE were determined by first testing a range of concentrations on three samples

until absorbance values did not increase. Incubation times were increased if absorbance values were still low after the initial incubation time from the referenced protocols. Details of the final assays are presented in Table 1. 50 mM sodium acetate buffer pH 5.0 was used for all assays. All enzyme assays were performed in microplates and measured colourimetrically using an EZ Read 400 microplate reader (Biochrom Ltd., Cambridge, United Kingdom). Absolute enzyme activities were calculated as potential activity per gram of dry weight soil and specific enzyme activities were calculated by dividing absolute enzyme activities by microbial biomass C (Moorhead et al. 2013).

Table 1. Details of the soil enzyme activity assays conducted in this study

Enzyme	Code	Related nutrient cycle	Substrate	Slurry concentration	Incubation time (h)
Phenol oxidase	POX	C	L-3,4-dihydroxyphenylalanine (20 mM)	0.5 g soil + 25 mL buffer	20
Lignin peroxidase	PER	C	L-3,4-dihydroxy phenylalanine and H_2O_2 (20 mM)	0.5 g soil + 25 mL buffer	1.5
Urease	URE	N	Urea (1 M)	2 g soil + 5 mL buffer	2
ß-glucosidase	GLC	C	*p*-nitrophenyl-*β*-D-glucopyranoside (30 mM)	3.75 g + 5 mL buffer	2.5
ß -xylosidase	XYL	C	*p*-nitrophenyl-*β*-D-xylopyranoside (25 mM)	3.75 g + 5 mL buffer	7
N-acetyl-*β*-glucosaminidase	NAG	C and N	*p*-nitrophenyl- N-acetyl-*β*-D-glucosaminide (5 mM)	3.75 g + 5 mL buffer	6
Cellobiohydrolase	CBH	C	*p*-nitrophenyl-*β*-D-cellobioside (4 mM)	3.75 g + 5 mL buffer	8
Acid phosphatase	AP	P	*p*-nitrophenyl-phosphate disodium salt hexahydrate (30 mM)	3.75 g + 5 mL buffer	0.5

2.5 Bacterial community diversity and composition

A metabarcoding approach was used to examine soil bacterial community diversity and composition. DNA was extracted the day after the end of the experiment from 0.5 g fresh weight soil from three of the five mesocosms within each treatment group using the PowerSoil PowerLyser DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). An extraction blank containing no soil was also included to identify contamination during the extraction process. DNA yield and quality were checked using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A paired-end dual-index sequencing approach was used. The V4 region of the 16S rRNA gene was amplified with primers 515F (5'-

GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') from Caporaso et al. (2011) containing Illumina adapter sequences and 8 bp index sequences following a one-step PCR protocol (Kozich et al. 2013; Griffiths et al. 2018). PCRs were performed in 15 µL reactions containing 20 ng DNA, 1 x KAPA HiFi HotStart ReadyMix, and 0.2 µM of each primer with the following cycle conditions: 95 °C for 5 min; followed by 28 cycles of 95 °C for 20 sec, 55 °C for 15 sec, 72 °C for 30 sec; followed by 5 min extension at 72 °C. All PCRs were conducted in duplicate and each plate contained a negative control as well as a mock community control (Microbial Mock Community B Staggered, Low Concentration, v5.2L for 16S Gene sequencing, HM-783D, BEI Resources, Manassas, VA, USA). PCR duplicates were pooled, then cleaned using Ampure XP beads (Beckman Coulter Inc., Indianapolis, IN, USA). Fragment size was checked on a 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Samples were normalised using a titration run, in which equal volumes of each sample were combined and sequenced using v2 nano (2 x 150bp) chemistry on a MiSeq (Illumina, Inc., SanDiego, CA, USA) at the University of Salford. Based on the number of reads assigned to each sample, an equimolar pool of all samples was created and sequenced on a MiSeq using 250 bp paired-end v2 chemistry.

Sequencing data were processed in R v3.5.2 (R Core Team 2018) with DADA2 v1.10.1 (Callahan et al. 2016) using the default pipeline. After paired-end reads had been merged, the modal sequence length was 253 bp. Ten sequence variants (SVs) of length >260 bp (0.002% of total sequences) and 992 bimeras were removed. Sequence variant taxonomies were mapped to genus level using the Silva v132 training set (Quast et al. 2013). 46% of SVs were unknown at the genus level so their assignments were edited from "NA" to their family name so they would not all be lost when later agglomerating to genus. Data were then exported to the phyloseq package (McMurdie & Holmes 2013) for further analysis.

Once eukaryota, archaea, chloroplast, and mitochondria reads had been removed, the number of assigned reads for each sample ranged from 15,606 to 29,600. Rarefaction curves were plotted using the rarecurve function in the vegan package (Oksanen et al. 2019), and these indicated that sampling depth was sufficient for all samples, plateauing at around 5,000 reads. The negative control did not contain any reads so no adjustments were made to the libraries to account for reagent contamination. To evaluate

245 the mock community results, precision, recall, and F-scores were calculated using the number of genera
246 correctly identified, the number of false positives, and the number of false negatives (Bokulish et al.
247 2018).

248 *2.6 Statistical analyses*

249 All analyses were conducted in RStudio v1.1.456 (RStudio Team 2016) for R v3.5.2 (R Core Team 2018)
250 unless otherwise stated. Soil variables, microbial biomass C, qCO_2, enzyme activities, and bacterial
251 diversity were analysed using three-way ANOVAs with the aov package to test for effects of oxygen, N
252 addition, PK addition, and their interactions. Box-cox transformations were applied to total P and some
253 enzymes to meet ANOVA assumptions (Tables S1, S3 and S4) and URE activity was transformed with
254 log(x+1). Microbial biomass C and qCO_2 were not improved by transformations so aligned rank ANOVAs
255 were also performed. The outcome did not vary between the two ANOVA methods and we have
256 presented the three-way ANOVA results from the unranked data (Table S1). Post-hoc pairwise contrasts
257 were performed using the LSMeans Contrast function in JMP v14.3.0 (SAS Institute Inc., Cary, NC, USA) to
258 test for differences between nutrient treatments within the same oxygen treatment. As pH can strongly
259 influence activity of many enzymes (Sinsabaugh et al. 2008), correlations between absolute enzyme
260 activity and soil pH were tested with the cor.test function in R's base stats package using Spearman's rank
261 correlations. Microbial respiration data were analysed with a linear mixed-effects model, using the lme
262 function in R package nlme (Pinheiro et al. 2018), with oxygen, N addition, and PK addition as fixed effects
263 and sample nested within day of measurement as a random effect representing repeated measures
264 within each mesocosm. An ANOVA table was calculated with Wald chi-square tests for the model using
265 the Anova function in the car package (Fox & Weisberg 2011). Soil variables are presented as means ± 1
266 standard error through the manuscript.

267 For bacterial diversity analyses, sequencing libraries were rarefied to the lowest depth (11,993). Shannon
268 and Simpson diversity indices were calculated and plotted using the estimate richness and plot richness
269 functions in phyloseq (McMurdie & Holmes 2013). Diversity analyses were repeated on rarefied datasets
270 in which taxa with fewer than 10 or 100 total reads had been removed, but this did not affect the results

(data not presented). To assess composition differences between treatments, read counts were transformed to centred log-ratio (Aitchison 1986) using the transform function in the microbiome package (Lahti et al. 2017). Euclidean distances were calculated using the distance package in phyloseq (McMurdie & Holmes 2013) and the adonis function in vegan (Oksanen et al. 2019) was used to perform permutational multivariate analysis of variance (PERMANOVA) with 1,000 permutations to test effects of oxygen, N addition, and PK addition on bacterial community composition. Differences between samples were visualised using principal component analysis (PCA) performed with the rda function in vegan (Oksanen et al. 2019). The envfit function in vegan was also used to find significant soil variables to fit to the PCA plot as vectors. Homogeneity of treatment group dispersion (variance) was measured using the betadisper function and differences between groups were tested using the permutest.betadisper function, both in the vegan package (Oksanen et al. 2019).

Indicator analysis was performed using the multipatt function in the indicspecies package (De Cáceres & Legendre 2009) to identify bacterial taxa associated with nutrient addition. This method uses the relative abundance of a taxa and its relative frequency of occurrence within treatment groups to calculate an indicator value index. The significance of the association between the taxa and the treatment group is then tested using permutation tests (De Cáceres & Legendre 2009). To focus on indicators of nutrient effects, the two oxygen treatments were analysed separately. We tested for indicators within each individual nutrient treatment group as well as within specified group combinations (Control with +PK, +N with +NPK, +PK with +NPK). For this analysis, libraries were normalised using cumulative sum scaling (CSS; Paulson et al. 2013) due to the issues associated with using rarefied data for differential abundance analyses (McMurdie & Holmes 2014). This was performed using the phyloseq_transform_css function in the metagMisc package (Mikryukov 2019). To exclude rare taxa from the analysis, SVs with fewer than 100 reads or that were prevalent in fewer than two samples were removed. SVs were agglomerated at the genus level, except for SVs which had not been assigned to genus level which were agglomerated at the family level. CSS-normalised abundance heatmaps were produced for identified indicator taxa using the plot_heatmap function in phyloseq (McMurdie & Holmes 2013).

297 For enzymes that changed significantly with nutrient addition, the associate function in the microbiome
298 package (Lahti et al. 2017) was used to cross-correlate CSS-normalised taxa abundances with enzyme
299 activity using Spearman's rank correlations. As soil redox potential has been found to affect activity of
300 some enzymes associated with C, N, and P cycling (Pulford & Tabatabai 1988) it is possible that responses
301 of taxa could vary between oxygen environments. Therefore, the data were separated by oxygen
302 treatment for these correlations. Significance values were corrected for multiple testing using the false
303 discovery rate method (Benjamini & Hochberg 1995) and heatmaps of correlation coefficients significant
304 at the p-adjusted threshold of 0.05 were produced using the heat function in the microbiome package
305 (Lahti et al. 2017).

306 *2.7 Accession numbers*

307 Forward and reverse sequence reads have been deposited in the NCBI Sequence Read Archive under
308 BioProject ID PRJNA632865. All other data will be archived in the Mendeley Data repository.

309 **3. Results**

310 *3.1 Soil analyses*

311 Experimental treatments resulted in significant differences in some of the measured soil variables (Table
312 2, Table S1). Addition of PK increased total soil P by 7.4 % ($F_{1,32}$ = 35.90, $p < 0.001$), with no significant
313 difference between the oxygen treatments ($F_{1,32}$ = 0.83, $p = 0.368$). There was a significant interaction
314 effect of oxygen treatment and N addition ($F_{1,32}$ = 21.23, $p < 0.001$) on total soil N, with N addition
315 resulting in larger N increases in ambient oxygen (+24.7 %) compared to reduced oxygen (+10.1 %). The
316 oxygen environment ($F_{1,32}$ = 136.17, $p < 0.001$) and N addition treatment ($F_{1,32}$ = 19.12, $p < 0.001$) had
317 significant effects on soil pH, with the average pH in reduced oxygen mesocosms 12.6 % higher than in
318 the ambient, and average pH in N addition mesocosms 4.5 % higher than in those without N addition.
319 Total soil C (22.52 % ± 0.16), moisture content (82.7 % ± 0.05), and organic matter content (46.50 % ±
320 0.12) did not vary significantly with oxygen or nutrient treatments (Table S1).

321 *3.2 Microbial biomass and activity*

Oxygen levels within the mesocosms had a significant effect on microbial respiration (Table 3) with higher values recorded in the reduced oxygen treatment (Figure 1). Nutrient additions did not significantly affect microbial respiration (Table S2), but the +NPK treatment tended to have higher respiration rates than the other treatments in the reduced oxygen mesocosms (Figure 2). This trend was not observed in the ambient oxygen incubations. There was a significant interaction effect of oxygen treatment and N addition ($F_{1,32} = 10.97$, $p = 0.002$, Table S1) on microbial biomass, with lower microbial biomass in the N addition treatments compared to the control in the reduced oxygen treatment only (Figure 2a). When microbial respiration was considered per unit microbial biomass (i.e. qCO_2), there was a significant interaction effect of oxygen treatment and N addition ($F_{1,31} = 20.47$, $p < 0.001$, Table S1) with qCO_2 increasing with N addition in the reduced oxygen environment only (Figure 2b).

Table 2. Mean (± 1 standard error) measures for soil variables at the end of experiment in ambient and reduced oxygen environments with N and PK addition treatments

Oxygen environment	Nutrient treatment	Total P %	Total N %	Total C %	pH	Organic Matter %	Moisture % wet weight
Reduced	Control	0.068 ± 0.001 a	1.33 ± 0.02 a	22.52 ± 0.49	7.69 ± 0.2 a	46.35 ± 0.51	82.86 ± 0.05
	+ PK	0.074 ± 0.001 b	1.34 ± 0.02 ab	22.58 ± 0.29	7.78 ± 0.15 a	46.17 ± 0.41	82.82 ± 0.08
	+ N	0.068 ± 0.002 a	1.52 ± 0.04 c	22.83 ± 0.24	7.95 ± 0.11 ab	46.67 ± 0.32	82.76 ± 0.12
	+ NPK	0.072 ± 0.001 b	1.42 ± 0.04 b	21.48 ± 0.51	8.12 ± 0.02 b	46.31 ± 0.2	82.84 ± 0.08
Ambient	Control	0.068 ± 0.0003 a	1.35 ± 0.04 a	22.3 ± 0.72	6.83 ± 0.05 a	46.04 ± 0.25	82.51 ± 0.15
	+ PK	0.072 ± 0.002 b	1.37 ± 0.01 a	22.75 ± 0.31	6.80 ± 0.07 a	46.41 ± 0.29	82.75 ± 0.32
	+ N	0.067 ± 0.001 a	1.68 ± 0.02 b	22.74 ± 0.33	7.18 ± 0.08 b	46.39 ± 0.38	82.61 ± 0.14
	+ NPK	0.072 ± 0.0005 b	1.71 ± 0.04 b	23 ± 0.41	7.19 ± 0.05 b	47.18 ± 0.18	82.71 ± 0.17

When significant effects of nutrient addition were found, posthoc tests were performed to test for differences between nutrient addition treatments within the same oxygen treatment (results indicated by different lowercase letters)

337

338 Figure 1. Microbial respiration response to nutrient addition in ambient and reduced oxygen mesocosms.
339 Data displayed are means ± 1 standard error and are offset across the X axis to minimise overlap.

340

341 Figure 2. Effect of oxygen environment and nutrient addition on a) microbial biomass carbon (MBC) and
342 b) microbial metabolic quotient (qCO_2) across oxygen environments and nutrient addition treatments.
343 The boxes show the median between the first and third quartiles and the whiskers extend up to 1.5 x the
344 interquartile range.

345 Absolute activities of all enzymes, except for POX and CBH, were significantly lower in the reduced oxygen
346 mesocosms (Figure 3; Table S3). Nitrogen addition resulted in significant decreases in absolute activity of
347 PER ($F_{1,32} = 5.22$, $p = 0.029$), URE ($F_{1,32} = 26.32$, $p < 0.001$), and AP ($F_{1,32} = 20.76$, $p < 0.001$). The only
348 enzyme whose absolute activity was significantly affected by PK addition was PER ($F_{1,32} = 1.19$, $p = 0.042$),
349 but post-hoc tests did not find any differences between PK addition treatments and the control in either
350 oxygen environment. Soil pH had significant negative correlations with the absolute activity of PER
351 (Spearman's Rho = -0.393, $p = 0.012$), URE (Spearman's Rho = -0.571, $p < 0.001$), GLC (Spearman's Rho = -
352 0.477, $p = 0.002$), NAG (Spearman's Rho = -0.691, $p < 0.001$), and AP (Spearman's Rho = -0.883, $p <$
353 0.001). When enzyme activities were considered per unit microbial biomass, there was a significant
354 interaction of oxygen and N addition on specific activities of PER ($F_{1,31} = 4.76$, $p = 0.037$), POX ($F_{1,31} = 10.11$,
355 $p = 0.003$), CBH ($F_{1,31} = 5.64$, $p = 0.024$), GLC ($F_{1,31} = 11.25$, $p = 0.002$), XYL ($F_{1,31} = 15.39$, $p < 0.001$), NAG
356 ($F_{1,31} = 19.81$, $p < 0.001$), and AP ($F_{1,31} = 12.97$, $p = 0.001$), all of which increased with N addition in the
357 reduced oxygen environment only (Figure S1, Table S4). Specific activity of URE decreased with N addition
358 ($F_{1,31} = 15.72$, $p < 0.001$) regardless of oxygen environment.

359

360 Figure 3. Absolute soil enzyme activity for each treatment group, measured at the end of the experiment.
361 Bars show mean ± 1 standard error and different lowercase letters indicate significant difference between

nutrient treatments within the same oxygen treatment at $p \leq 0.05$ using post-hoc pairwise contrasts. All values are untransformed data expressed on a dry weight basis.

3.3 Soil bacterial community diversity and composition

Sequence variants (SVs) were assigned to 52 unique phyla with three phyla accounting for 75.9% of the reads that were able to be assigned at the Phylum level (Proteobacteria, Bacteroidetes, Epsilonbacteraeota; Figure 4). At the phylum level, N addition increased relative abundance of Epsilonbacteraeota in the ambient oxygen mesocosms, and increased relative abundance of Proteobacteria in the reduced oxygen mesocosms. Across all samples, 37.4% of SVs were shared between the ambient and reduced oxygen mesocosms. Within the ambient oxygen mesocosms, 38.7% of SVs were shared between samples with and without N addition. Within the reduced oxygen mesocosms, there were greater differences between the samples with and without N addition, with only 33.7% of shared SVs. The three most abundant genera in the reduced oxygen mesocosms were *Arcobacter* (8.2% of reads), *Sulfurimonas* (5.9%), and *Marinobacter* (5.0%). The three most abundant genera in the ambient oxygen mesocosms were *Arcobacter* (7.7%), *Ignavibacterium* (3.5%), and *Marinobacterium* (3.4%). 68% of SVs were unable to be assigned at genus level. Of the 17 genera expected in the mock community sample, 15 were detected. The two genera that were not detected were *Propionibacterium* and *Actinomyces* and these were only expected in low concentrations. There were no false positives and the F-score was 0.94.

Figure 4. Relative abundances of bacterial phyla across oxygen and nutrient addition treatments. Only phyla with relative abundances above 1% are plotted.

Bacterial diversity was significantly reduced with N addition in both the ambient and reduced oxygen mesocosms (Figure 5, Table 3), and was lower in the reduced oxygen mesocosms compared to the ambient ones. There were significant effects of oxygen environment and N addition on bacterial

community composition, and a significant interaction was detected between the two (Table 4). Oxygen environment and N addition each accounted for ~14% of the variation in bacterial community composition. These effects are illustrated in Figure 6, where control and +PK samples group together, and +N and +NPK samples group together in both oxygen treatments, which are themselves distinct groups. Total N content (R^2 = 0.77, $p < 0.001$) and pH (R^2 = 0.80, $p < 0.001$) of the soil were both found to have significant associations with the bacterial community composition. There was no difference in multivariate dispersions between the treatment groups ($F_{7, 16}$ = 0.93, p = 0.522).

Figure 5. Effects of nutrient addition on the diversity of soil bacterial communities after 15 days incubation in ambient or reduced oxygen environments. Boxplots of Shannon and Simpson Indices determined from rarefied 16S rRNA gene sequencing data. The boxes show the median between the first and third quartiles and the whiskers extend up to 1.5 x the interquartile range.

Table 3. Three-way ANOVA results testing effects of oxygen environment, N addition, and PK addition on bacterial community diversity in soil, using Shannon and Simpson diversity indices calculated from rarefied 16S rRNA gene sequencing data.

	Shannon		**Simpson**	
	$F_{1,16}$	p-value	$F_{1,16}$	p-value
Oxygen	59.03	<0.001	16.80	<0.001
N	90.55	<0.001	45.26	<0.001
PK	2.03	0.174	0.01	0.921
Oxygen:N	0.24	0.632	0.34	0.566
Oxygen:PK	2.27	0.152	2.51	0.133
N:PK	0.20	0.661	1.19	0.292
Oxygen:N:PK	0.96	0.342	0.66	0.430

Table 4. Results of Permutational Multivariate Analysis of Variance (PERMANOVA) to test for effects of oxygen environment, N addition, and PK addition on soil bacterial community composition.

	$F_{1,16}$	R^2	p-value
Oxygen	4.63	0.146	0.001
N	4.53	0.143	0.001
PK	1.20	0.038	0.187
Oxygen:N	1.98	0.038	0.008
Oxygen:PK	1.04	0.033	0.344
N:PK	1.10	0.035	0.279
Oxygen:N:PK	1.14	0.036	0.221

Figure 6. Principal components analysis (PCA) plot of soil bacterial communities from nutrient addition mesocosms incubated in ambient or reduced oxygen environments. PCA calculated from centred log-ratio transformed 16S rRNA sequencing data. Vectors of significant soil variables are plotted with grey arrows.

3.4 Bacterial indicators

The bacterial taxa that were identified as indicators for treatments with and without N addition are shown in Figures 7 and 8 (for further details see Tables S5 and S6). Only one taxa was identified as an indicator of a single treatment group (*Shewanella* in +NPK ambient oxygen), the rest were indicators of a combined N addition group (+N and +NPK) or a combined no N addition group (Control and +PK). In the ambient oxygen mesocosms, 26 taxa were identified as indicators of samples without N addition (Control and +PK treatments), and 30 taxa as indicators for samples with N addition (+N and +NPK treatments). In the reduced oxygen mesocosms, 35 taxa were identified as indicators of samples without N addition (Control and +PK treatments), and 18 taxa as indicators for samples with N addition (+N and +NPK

treatments). No taxa were identified as indicators of a combined PK addition group (the +PK and +NPK treatments) in either oxygen environment. In both oxygen treatments, N addition drives a decrease in the overall abundance (Figure 9) of the sulfate-reducing order Desulfobacterales (Kuever et al. 2005), with eight taxa in this order identified as indicators of the group without N addition in the reduced oxygen and six taxa in the ambient oxygen. The N addition group saw an increase in abundance of several taxa of unknown function from the order Oceanospirallales (three taxa in reduced oxygen and five taxa in ambient oxygen). The only N-cycling related taxa that we identified amongst the indicators, was *Nitrococcus*, a nitrifying bacterium (Watson & Waterbury 1971) that increased abundance with N addition in the ambient oxygen treatment.

Figure 7. Heatmap showing cumulative sum scaling (CSS) normalised abundances of soil bacteria indicator taxa identified for treatments with and without N addition in ambient oxygen mesocosms. Only taxa significant at $p \leq 0.01$ are shown. For taxa where genus could not be assigned, the family is shown instead.

Figure 8. Heatmap showing cumulative sum scaling (CSS) normalised abundances of soil bacteria indicator taxa identified for treatments with and without N addition in reduced oxygen incubations. Only taxa significant at $p \leq 0.01$ are shown. For taxa where genus could not be assigned, the family is shown instead.

Figure 9. Total abundances for bacteria from the sulfate-reducing order Desulfobacterales within each nutrient addition treatment group in ambient and reduced oxygen mesocosms. Each section within a bar is the number of sequencing reads of the 16S rRNA gene for one sample using rarefied data.

3.5 Correlations between bacterial abundance and enzyme activities

444 In the ambient oxygen mesocosms, no significant correlations were found between abundances of
445 bacterial genera and absolute soil enzyme activities. In the reduced oxygen mesocosms, 33 taxa
446 significantly correlated with URE, nine taxa correlated with AP, and only one taxa correlated with PER
447 activity (Figure 10). Six taxa significantly correlated with both URE and AP, and all of these were negative
448 correlations. There was some overlap between the indicator taxa and those that significantly correlated
449 with enzyme activities. Eleven of the indicator taxa for the reduced oxygen treatments with N, had
450 significant negative correlations with URE activity, and nine of the indicator taxa for the reduced oxygen
451 treatments without N had significant positive correlations with URE.

452

453 Figure 10. Heatmap showing Spearman correlation coefficients for taxa that significantly correlated (FDR
454 p adjusted value ≤ 0.05) with absolute enzyme activity of lignin peroxidise (PER), urease (URE), or acid
455 phosphatase (AP). For taxa where genera could not be assigned, the lowest assigned taxonomic rank is
456 shown. * also identified as indicator taxa

457 **4. Discussion**

458 Nitrogen addition drove clear shifts in bacterial community composition, and as hypothesised, resulted in
459 decreased bacterial diversity and decreased abundance of sulfate-reducing bacteria. These changes in
460 bacterial community structure were accompanied by changes in the activity of some enzymes involved in
461 C, N and P cycling. Although nutrient addition had no effect on overall microbial respiration, N addition
462 decreased microbial biomass and increased microbial metabolic quotient in the reduced oxygen
463 environment. The addition of PK alone did not have any significant effects on bacterial diversity or
464 composition, and soil functions were not altered when P and K were combined with N. Overall, these
465 results indicate that N addition causes shifts in the diversity and composition of mangrove soil bacterial
466 communities with potential consequences for nutrient cycling and decomposition processes.

467 In contrast to other mangrove soil nutrient addition experiments, where microbial respiration increased
468 with nutrient addition (Chen et al. 2011, Keuskamp et al. 2013, Davies et al. 2017), we did not see
469 significant responses of microbial respiration to either N or PK. However, when respiration was

considered per unit of microbial biomass (ie. qCO_2), an increase in qCO_2 with N addition was detected in the reduced oxygen environment, suggesting that it was the observed decrease in microbial biomass which contributed to the lack of an overall effect. This decrease in soil microbial biomass with N addition is consistent with the findings of many other studies across a range of biomes, which can have corresponding decreases in soil CO_2 emissions (Treseder 2008). Our findings suggest a change in the physiological status of the microbial community, with increased qCO_2 indicating reduced C use efficiency with N addition in the reduced oxygen soils. In field conditions, we must also consider the indirect effects of nutrient addition on microbial respiration and biomass through increased plant productivity leading to an increased supply of C to the soil microbial community via exudates (Uselman et al. 2000). While plant growth showed strong positive responses to N addition at the field site related to this study (Dangremond et al. 2019), microbial responses to nutrient addition in this incubation study were not so straightforward. Contrasting nutrient limitations are often observed between plants and their soil microbes, with organic carbon typically being the dominant limitation for secondary productivity by heterotrophic microbial communities (Soong et al. 2000). While mangrove soils are often rich in organic carbon, previous studies indicate that their microbial communities can be both C and N limited, with Chen et al. (2011) and Keuskamp et al. (2013) only detecting increases in microbial respiration with nutrient addition when a labile C source was also added.

Nitrogen addition generally suppresses activity of lignin-modifying enzymes such as lignin peroxidise (PER) and phenol oxidase (POX) leading to increases in soil C storage (Chen, Luo et al. 2018). Here, N addition suppressed absolute activity of PER, but did not affect absolute activity of POX. Our lack of response in absolute POX activity corresponds with the findings of another N addition incubation experiment of mangrove soils by Luo et al. (2017). We did, however, find an increase in specific activity of POX with N addition under reduced oxygen, and this is likely due to lower microbial biomass in the N addition treatments. Nitrogen addition has also been associated with increased activity of cellulases such as *ß*-glucosidase (GLC), *ß*-xylosidase (XYL), and cellobiohydrolase (CBH) (Chen, Luo et al. 2018) but no changes in absolute activity were observed in these three enzymes in this study. Previous N addition incubation experiments with mangrove soils have observed either an increase (Keuskamp et al. 2015) or a

497 decrease (Luo et al. 2017) in absolute GLC activity. Despite the lack of discernable response in the
498 absolute activity of GLC in our study, significant increases were observed in the specific activity of GLC
499 under reduced oxygen. Similar to what was observed with microbial respiration, these findings suggest N
500 addition may have resulted in a physiological response at the microbial level but the accompanying
501 decrease in microbial biomass contributed to the lack of an overall effect of N addition on GLC activity
502 within the reduced oxygen mesocosms. The variable responses observed here in enzymes involved in C
503 cycling make it difficult to predict how carbon storage rates might be affected.

504 Under the resource allocation model (Sinsabaugh & Moorhead 1994), N addition should result in
505 decreased activity of N acquiring enzymes. A marked decrease in absolute activity of urease (URE) was
506 observed with N addition in the reduced oxygen treatment, and while the same pattern was observed in
507 the ambient oxygen treatment, the response was more variable. In a previous experiment with mangrove
508 soils (Chen et al. 2015), N addition was found to have a stimulatory effect on soil URE activity (Chen et al.
509 2015). However, it should be noted that in Chen et al.'s (2015) study the positive effect of N was only
510 observed in soils that had plants growing in them, there was no effect of N on URE activity in the non-
511 planted soils. We observed no change in absolute activity of *N*-acetyl-*β*-glucosaminidase (NAG), but
512 specific activity of NAG was higher with N under reduced oxygen. Nitrogen addition has been found to
513 increase both absolute and specific activity of NAG in waterlogged rice paddies (Zhang et al. 2015) but in
514 other incubation experiments with mangrove soils, absolute activity of NAG has either decreased (Luo et
515 al. 2017) or been unaffected (Keuskamp et al. 2015). Keuskamp et al. (2015) also assessed activities of
516 two other N acquiring enzymes: leucine aminopeptidase (LAP) and glycine aminopeptidase (GAP). They
517 saw increased activity of LAP with N addition in incubation experiments but not in the field, and GAP did
518 not respond to N addition in the field or the incubations (Keuskamp et al. 2015). These conflicting results
519 amongst N-acquiring enzymes support the findings from Sinsabaugh & Follstad Shah's (2012) review
520 paper covering a range of ecosystems that there is no simple relationship between N availability and N
521 acquiring extracellular enzyme activity.

Contrary to our hypothesis and findings of other studies in terrestrial (Marklein 2012) and mangrove soils (Luo et al. 2017), absolute activity of the P acquiring enzyme acid phosphotase (AP) decreased with N addition rather than increased. We found a strong negative correlation between pH and AP activity, which we think accounts for the results that we observed, as pH significantly increased with N addition in our experiment. PER and URE also had significant negative correlations with pH suggesting that some of the enzyme responses that we are seeing could be driven by pH changes in the soil. This is similar to the findings of Liu & Zhang (2019), who found changes in enzyme activity with N addition were related more to changes in soil pH than changes in plant or microbial community composition, although in their study pH decreased rather than increased with N addition. Globally, pH tends to decrease with N addition, rather than increase as observed here, although responses can vary across ecosystem types (Tian & Niu 2015). Similar to our study, Luo et al. (2017) also observed an increase in pH with N addition in their incubation experiment with mangrove soils. We suggest that this increase, as well as the differences in pH between the oxygen treatments, could be related to the unique redox conditions found in wetland soils and the amount of oxidisable constituents present (D'Angelo & Reddy 2003). We suspect that pH changes of the magnitude observed in this incubation experiment would be unlikely in surface soils in the field due to regular tidal flushing. Significant effects of oxygen environment on absolute activity were observed in all enzymes except POX and CBH. This is not unexpected as many studies have found that waterlogging and redox potential can have significant effects on soil enzyme activities (eg. Pulford & Tabatabai 1988, Wang & Lu 2006, Vo & Kang 2013). Our results highlight the complexity of enzyme responses to nutrient amendment, and further research is required to assess enzyme responses and nutrient cycling processes under field conditions.

As hypothesised, soil bacterial diversity, measured by Shannon and Simpson diversity indices, decreased with N addition. Our findings are supported by other studies within mangroves (Wang et al. 2016), marine sediments (Aoyagi et al. 2015), and other terrestrial environments (Wang et al. 2018). Similar diversity decreases with N addition have also been observed in fungal (Wang et al. 2018) and plant communities (Midolo et al. 2019). There is currently no consensus on what drives this decrease in diversity, but it could be related to changes in competitive interactions, with fast-growing species that are able to take

advantage of increased N availability outcompeting slower growing oligotrophic species adapted to low nutrient conditions (Leff et al. 2015). Other studies have suggested that decreases in bacterial diversity with N are indirectly affected by changes in soil pH or plant communities (Zeng et al. 2016, Zhang et al. 2017). The loss of soil microbial diversity can lead to a reduction in soil functioning (Maron et al. 2018, Trivedi et al. 2019) so when considered alongside observed decreases in microbial biomass and the activity of some enzymes (e.g. PER, URE, AP), this suggests that nutrient cycling rates could be altered by N addition in this system.

Bacterial community composition was significantly affected by N addition. *Arcobacter* was the most abundant genus and an indicator taxa of N addition in both oxygen treatments. Species within this genus have previously been identified as indicators of fecal pollution in environmental water samples (Collado et al. 2008). This suggests that the mangrove site from which these soils were sourced may already be experiencing anthropogenic derived nutrient pollution but that nutrient concentrations were not saturated as N addition led to increased *Arcobacter* abundance. In both the ambient and reduced oxygen treatments, N addition resulted in decreased abundance of several genera within the sulfate-reducing order Desulfobacterales, and increased abundance of several genera from Oceanospirallales. This same trend was also reported in a nitrate-addition incubation study of saltmarsh sediment (Bulseco et al. 2019), which found that although sulfate-reduction remained the dominant process, a shift towards nitrate-reduction allowed for decomposition of organic matter that had previously been inaccessible in sulfate-reducing conditions. Bulseco et al. (2019) suggested that N induced changes in bacterial community composition could potentially increase decomposition rates as new pools of organic matter become available. While litter decomposition rates over six months did not vary with nutrient addition at the field experiment site associated with our study (Simpson et al. 2020), decomposition rates of older, more recalcitrant carbon sources have yet to be assessed. In contrast to previous studies (Aoyagi et al. 2015, Wang, et al. 2016, Bulseco et al. 2019) we did not observe increased abundance of nitrate-reducing bacteria such as *Sulfurimonas*. This genus actually decreased in abundance with N addition in the reduced oxygen treatment. Of the bacterial taxa whose abundance increased in our study, their functions were generally unknown, so we were unable to confirm a shift towards nitrate-reduction in our study. Further

work is needed in the field to assess changes in soil bacterial community structure and function and their potential impacts on nutrient cycling and decomposition in the longer term.

Changes in bacterial community composition drove changes in the activity of some enzymes associated with N addition. PER activity only correlated with abundance changes in one taxa, but 37 genera were identified whose relative abundance correlated with changes in URE and/or AP activity. As correlation does not imply causation, further work is needed to confirm direct linkages between the function of each of these taxa and their response to nutrient addition. Due to the lack of available functional information for many mangrove bacterial taxa, we support the suggestion of Trivedi et al. (2016) that abundances of functional genes may prove more useful than taxonomic abundance for predicting changes in enzyme activity and soil processes.

While this study has improved our understanding of microbial responses to nutrient addition in mangrove soils, there were some limitations associated with this study that may have influenced the findings. Microbial respiration was significantly lower in the ambient oxygen treatment compared to the reduced oxygen mesocosms. As microbial respiration is generally lower in anaerobic conditions (Reddy and DeLaune, 2008), we suggest that the high soil moisture content in the mesocosms provided a poor environment for aerobic microbes, but that the oxygen levels in the ambient mesocosms were sufficient to inhibit obligate anaerobic microbes. As such, responses of microbes in our ambient mesocosms to N addition may have been muted in comparison with the responses observed in the reduced oxygen mesocosms. Future mangrove incubation experiments could potentially improve aerobic conditions by increasing the soil surface area in the mesocosms relative to volume. Addition of PK had no significant effect on any of the measured microbial response variables. This could be because the microbial community in this soil was not P limited, but it could also be that the amount of P added was insufficient to induce a microbial response. This study targeted bacteria for community characterisation but the microbial activities measured would include responses from other components of the microbiome, such as archaea and fungi. Further research is needed to determine how these different kingdoms contribute to the overall response. We acknowledge that soil disturbance during the experimental setup may have

altered bacterial community composition in the mesocosms compared to the field. Future nutrient incubation experiments may wish to consider the use of intact cores or characterising the microbial community at different time points, over a longer duration, to track community changes and resilience.

4.2 Conclusions

This study highlights the potential vulnerabilities of mangrove microbial communities to N enrichment. We found that N addition caused significant shifts in the diversity and composition of soil bacterial communities and impacts on microbial biomass and functioning. This has potential implications for C and nutrient cycling which warrant further investigation in the field. While many mangrove habitats have already been subjected to increasing nutrient levels over the last few decades, additional nutrients may not elicit much response. However, some relatively pristine habitats still remain whose water catchments will see increased human impacts in the coming years. The findings from this study contribute to a better understanding of the effects of elevated nutrients on mangrove soil microbial communities which could help focus management of these environments more effectively.

5. Author Contributions

HC, LTS, and TZO conceived the study, and HC and LTS designed and conducted the experiment. HC, IC, and DA performed nutrient analyses and enzyme assays, HC and REA performed microbial sequencing work, and HC analysed the data. JKR, RDB, CHR, and TZO supervised the project and provided guidance on the framing of questions and interpretation of findings. HC prepared the manuscript with all authors contributing to the drafts.

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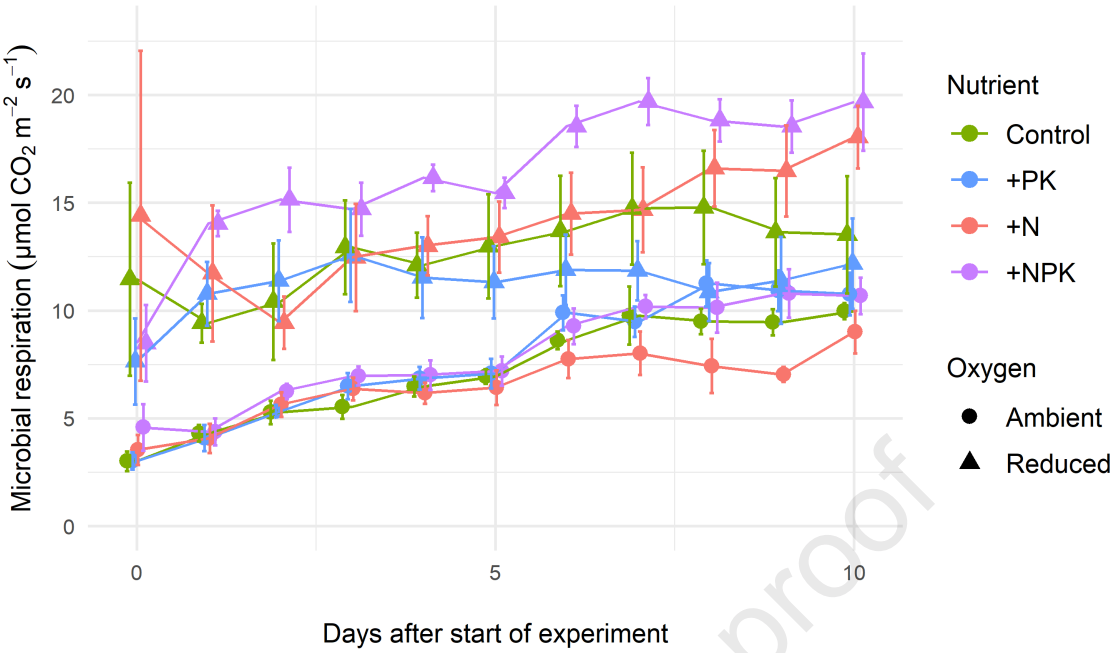
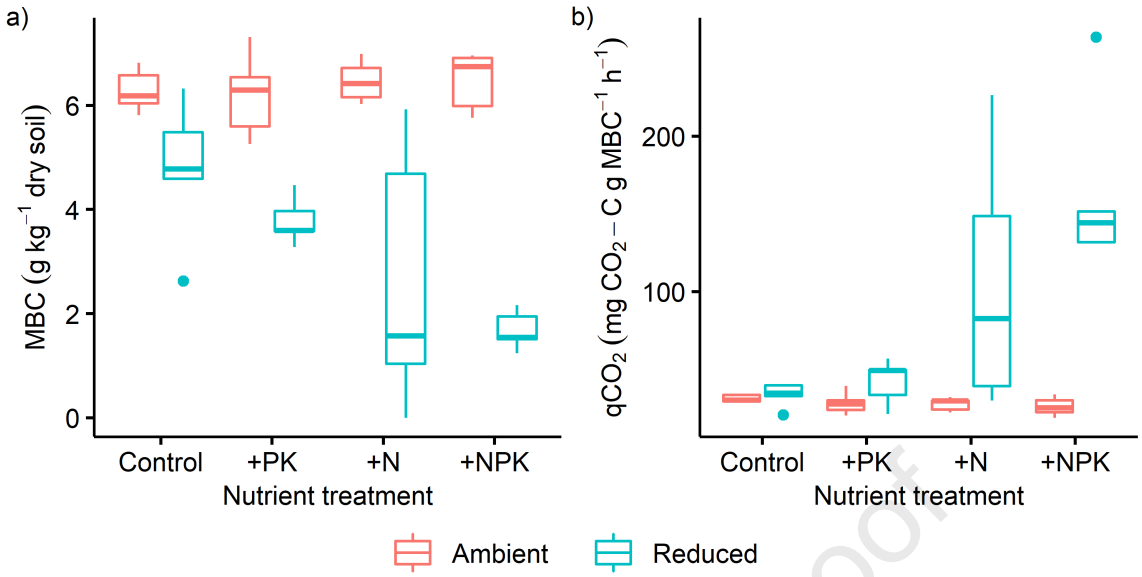
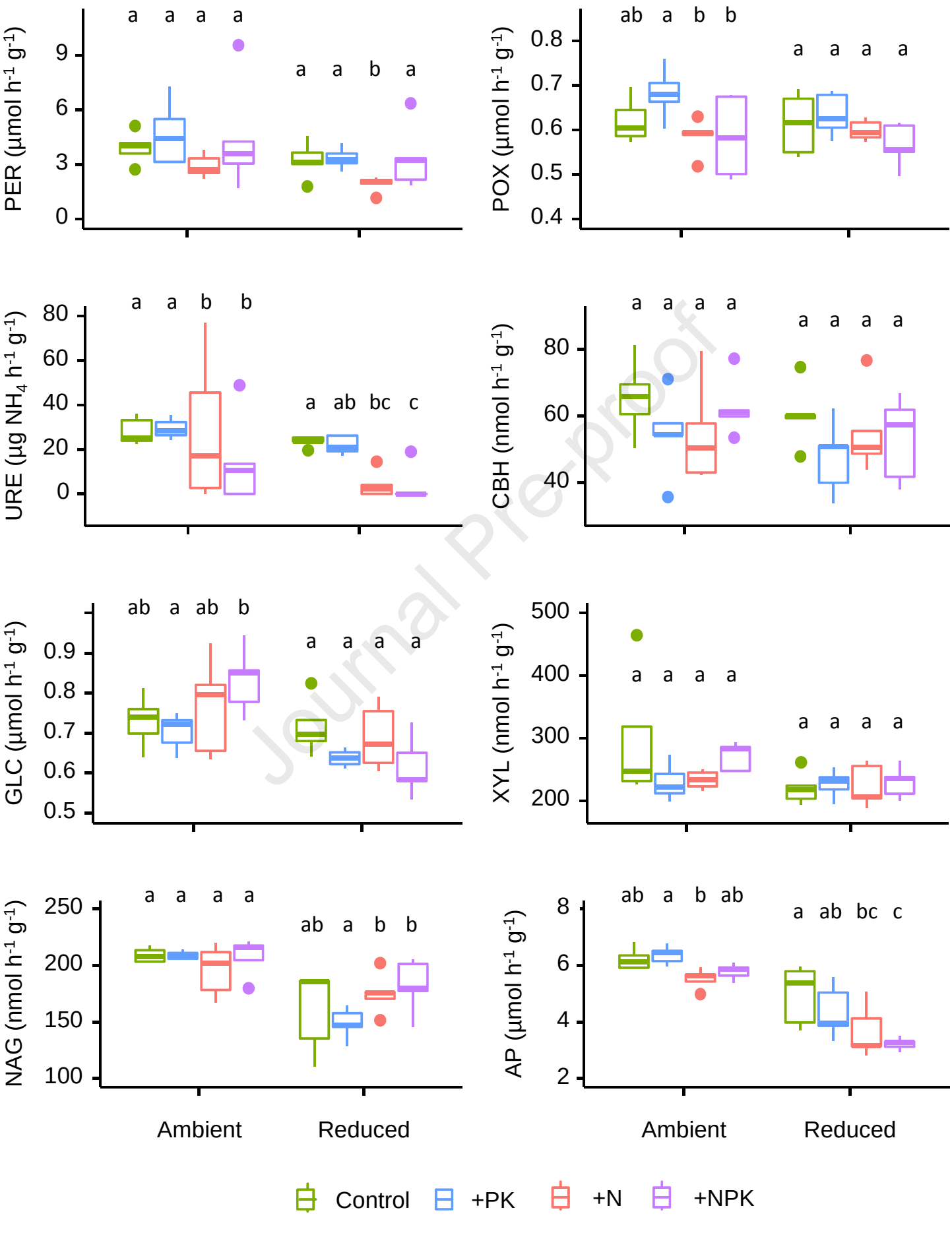
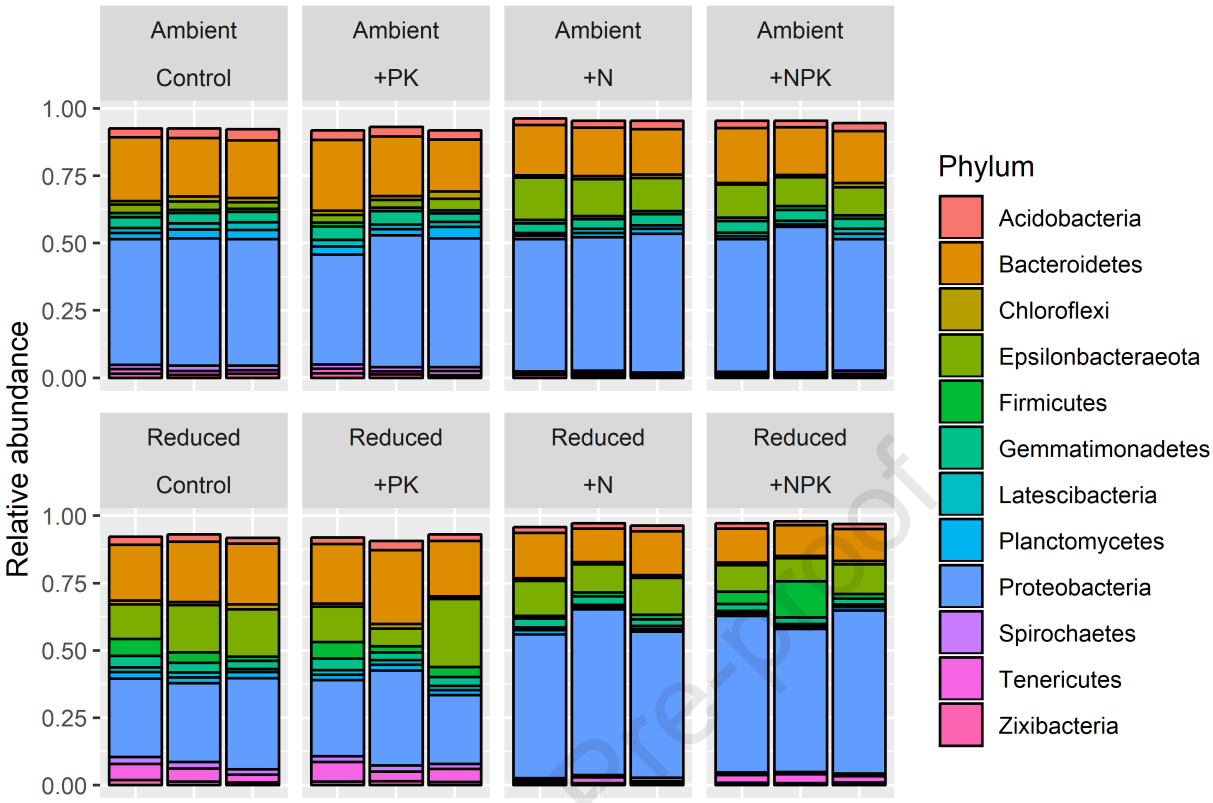
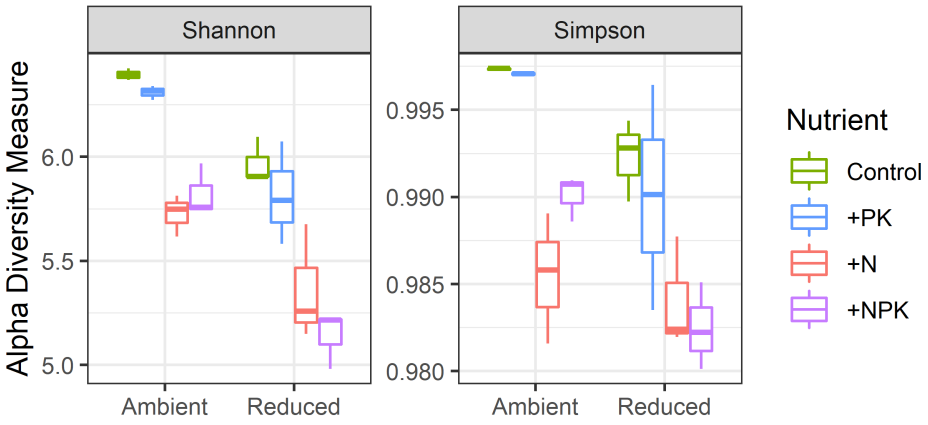
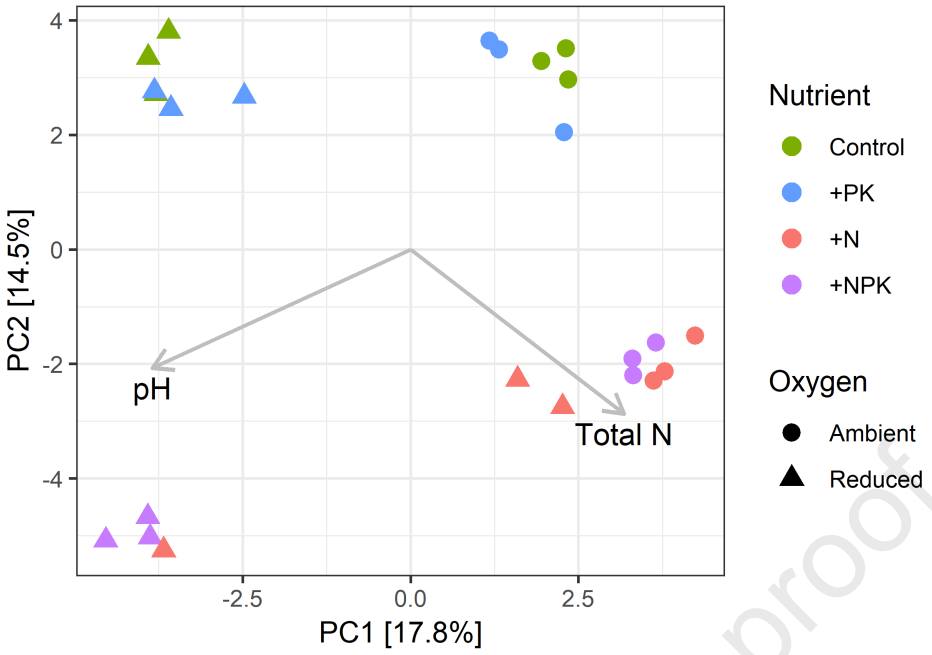


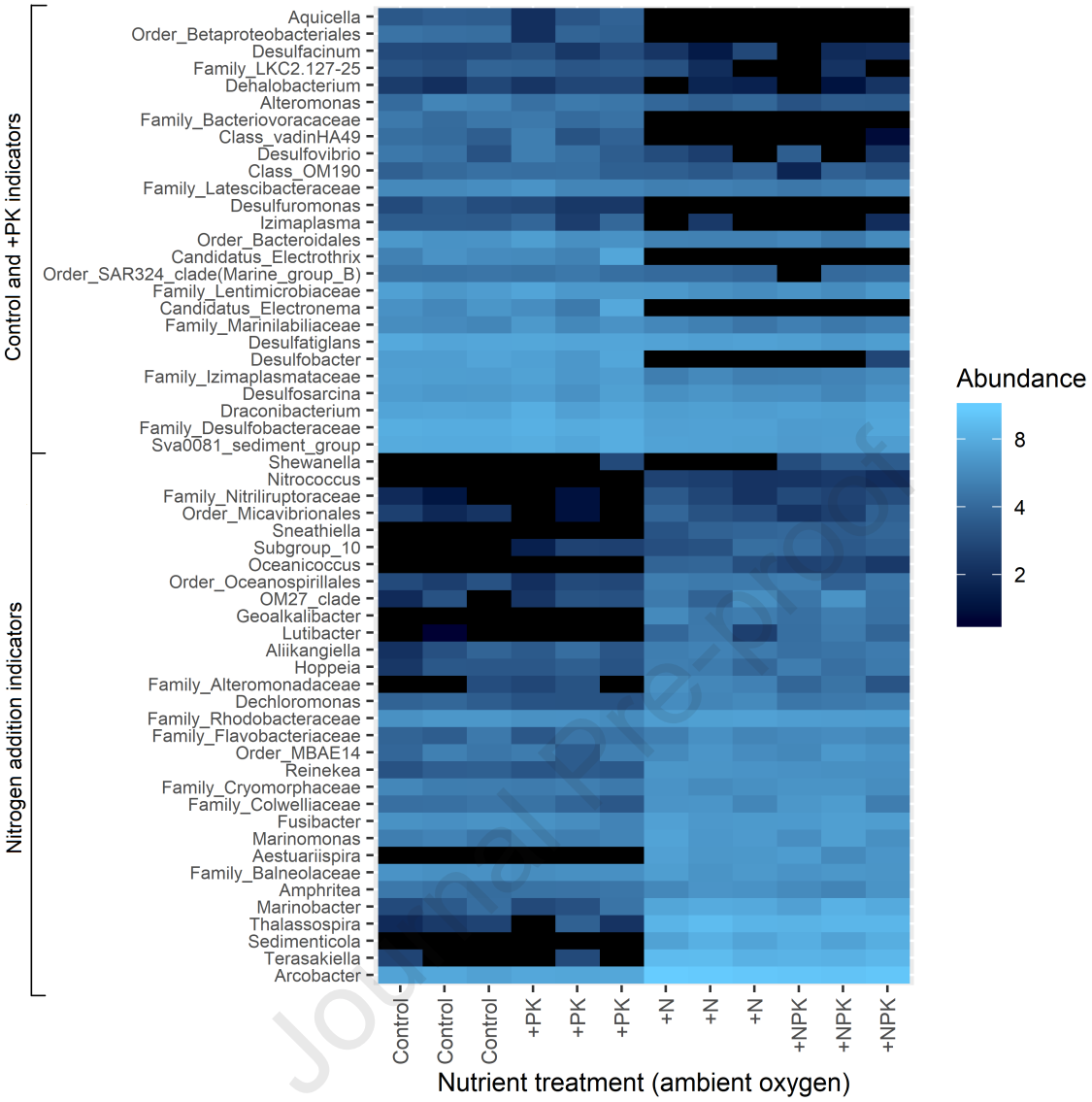


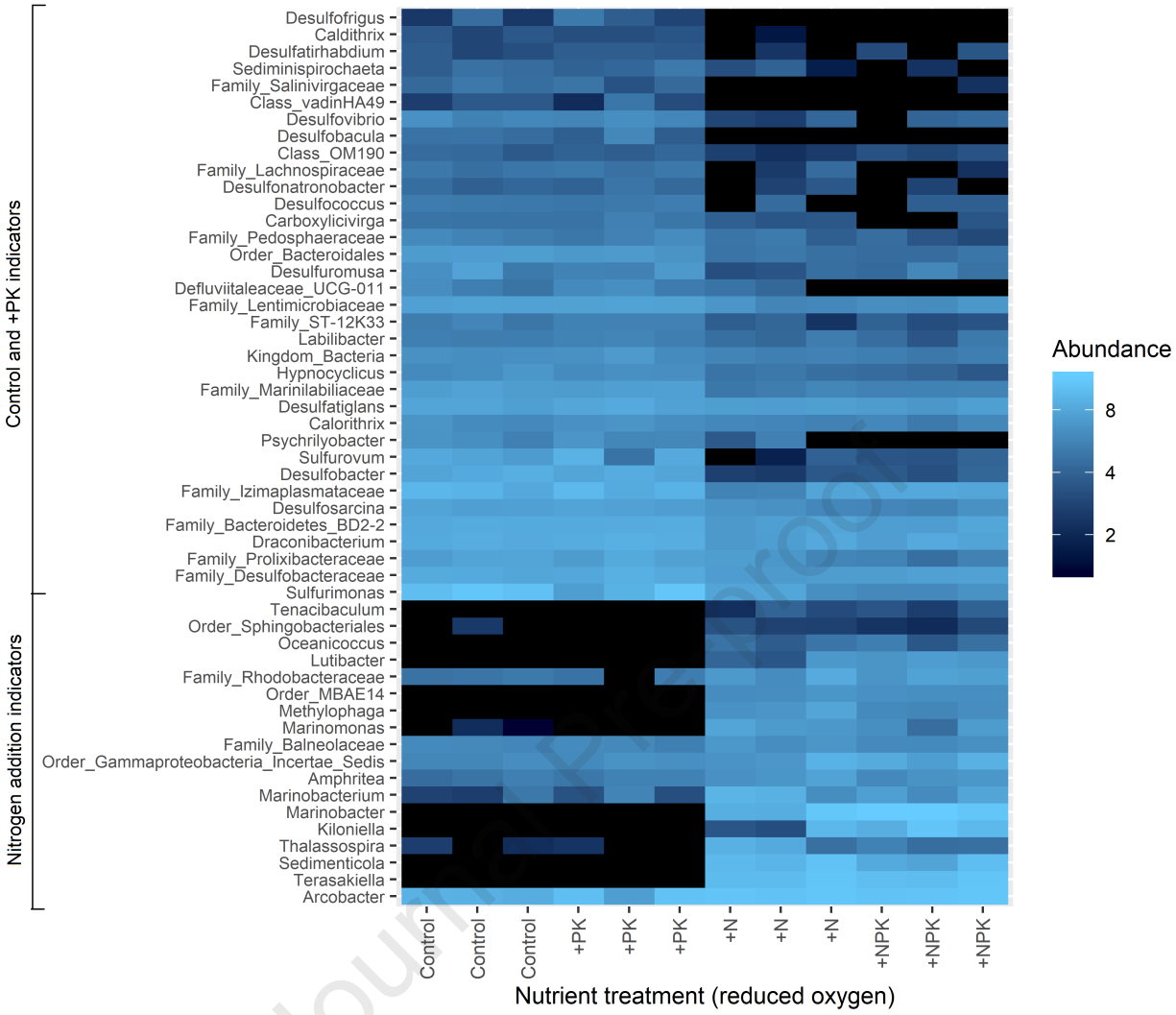


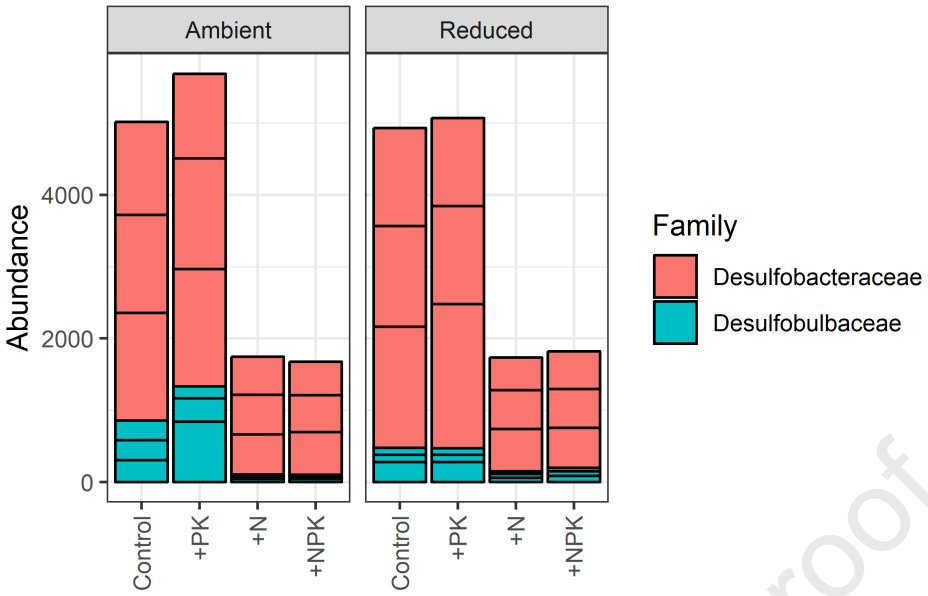


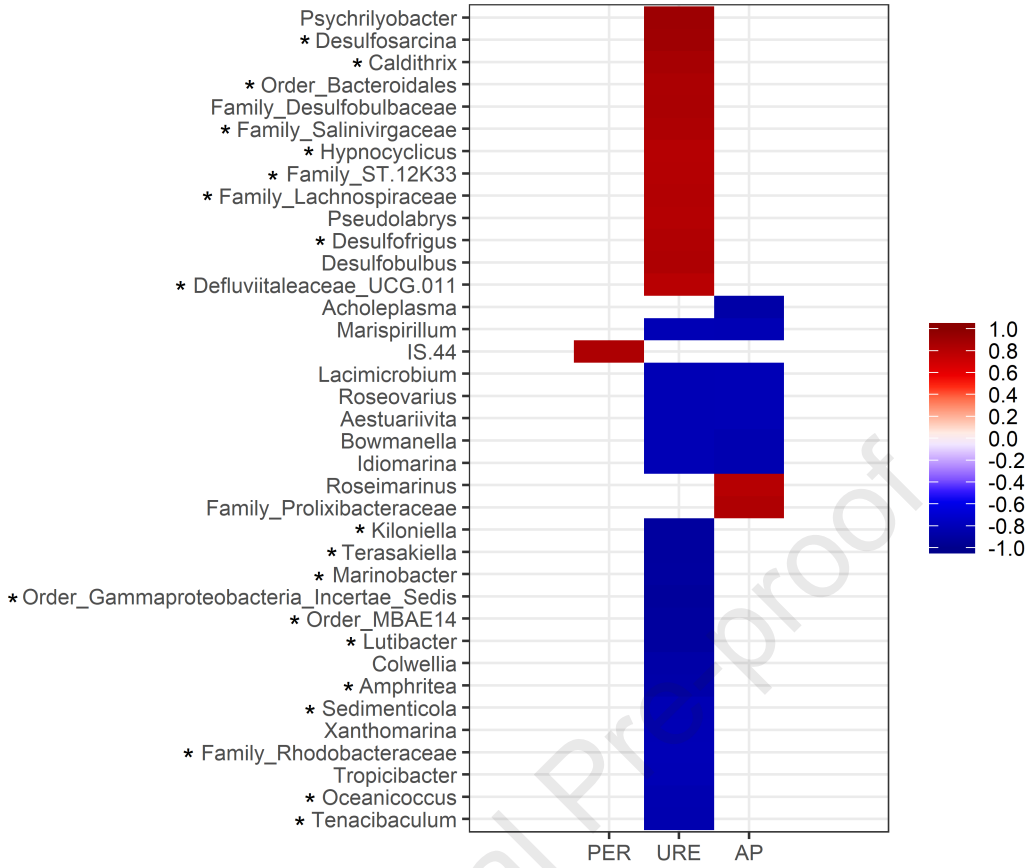





Control and +PK indicators
Nitrogen addition indicators
Aquicella
Order_Betaproteobacteriales
Desulfacinum
Family_LKC2.127-25
Dehalobacterium
Alteromonas
Family_Bacteriovoracaceae
Class_vadinHA49
Desulfovibrio
Class_OM190
Family_Latescibacteraceae
Desulfuromonas
Izimaplasma
Order_Bacteroidales
Candidatus_Electrothrix
Order_SAR324_clade(Marine_group_B)
Family_Lentimicrobiaceae
Candidatus_Electronema
Family_Marinilabiliaceae
Desulfatiglans
Desulfobacter
Family_Izimaplasmataceae
Desulfosarcina
Draconibacterium
Family_Desulfobacteraceae
Sva0081_sediment_group
Shewanella
Nitrococcus
Family_Nitriliruptoraceae
Order_Micavibrionales
Sneathiella
Subgroup_10
Oceanicoccus
Order_Oceanospirillales
OM27_clade
Geoalkalibacter
Lutibacter
Aliikangiella
Hoppeia
Family_Alteromonadaceae
Dechloromonas
Family_Rhodobacteraceae
Family_Flavobacteriaceae
Order_MBAE14
Reinekea
Family_Cryomorphaceae
Family_Colwelliaceae
Fusibacter
Marinomonas
Aestuariispira
Family_Balneolaceae
Amphritea
Marinobacter
Thalassospira
Sedimenticola
Terasakiella
Arcobacter
Control
Control
Control
+PK
+PK
+PK
+N
+N
+N
+NPK
+NPK
+NPK
Nutrient treatment (ambient oxygen)
Abundance
8
4
2







Highlights

- Nitrogen addition changed bacterial community composition and decreased diversity
- Nitrogen addition decreased abundance of sulfate-reducing bacteria
- Microbial biomass decreased and metabolic quotient increased with nitrogen addition
- Activity of enzymes involved in decomposition changed with nutrient addition

Declaration of interests

☑ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: